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Erythropoietin Receptor and Hematological Disease

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This review will discuss evidence for the role of the erythropoietin (Epo) receptor in the development of erythrocytosis and other hematological disorders. The possible causative role of mutations of other genes in the pathogenesis of idiopathic erythrocytosis will be considered. Polycythemia vera (PV) is a myeloproliferative disorder that is caused by an undefined stem cell abnormality, characterized by a significant erythrocytosis, leukocytosis, and thrombocytosis. However, erythrocytosis may arise from apparent (or relative) polycythemia in which the hematocrit is raised due to a low plasma volume. In such cases the red cell mass is normal. A group of disorders with increased red cell mass caused by stimulation of erythrocyte production is known as secondary polycythemia. Investigation of such patients may reveal a congenital abnormality such as high affinity hemoglobin or an acquired abnormality caused, for example, by smoking, renal vascular impairment, or an Epo-producing tumor. Even after thorough examination there remains a cohort of patients for whom no definite cause for the erythrocytosis can be established. A careful clinical history may reveal whether this idiopathic erythrocytosis is likely to be congenital and/or familial, in which case the term ''primary familial and congenital polycythemia'' is sometimes applied. Access to a range of laboratory investigations may define the molecular pathophysiology. We will now discuss how this process can be investigated. Am. J. Hematol. 60:55–60, 1999. **© 1999 Wiley-Liss, Inc.**

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Erythropoietin

Erythropoietin (Epo) is the lineage-specific regulator required for survival, proliferation, and differentiation of committed erythroid progenitor cells [for reviews see 1,2]. It is synthesized by the kidney under the control of a feedback mechanism. At low-oxygen tensions the synthesis of Epo is stimulated resulting in increased erythroid cell production. In the adult, under steady-state conditions, 2.3×10^6 red cells per sec are produced and the Epo concentration is in the picomolar range corresponding to 5–20 mU/ml. In anemia, a wide range of Epo levels are found depending on the cause of the anemia. This varies from 10 mU/ml in chronic renal failure to values that are frequently in excess of 1,000 mU/ml in aplastic anemia [3]. With erythrocytosis the Epo level can be increased or paradoxically decreased. In some secondary cases the increased level is dependent on the cause, for example an Epo-secreting tumor. However, the idiopathic or primary cases can have an increased or decreased level of Epo. Some of the cases in which the level is decreased have been explained by a recently described disorder of the erythropoietin receptor (EpoR).

The Erythropoietin Receptor

The EpoR is located on the surface of erythroid cells and undergoes phosphorylation in response to Epo [4,5]. The receptor consists of 508 amino acids in the human (507 in the mouse) and consists of an extracellular domain, a single hydrophobic transmembrane domain, and a cytoplasmic domain [6]. It belongs to a superfamily of cytokine receptors which includes receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF),

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interleukin (IL)-3, and IL-6 [7]. In common with other members, there are four conserved cysteine residues and a WSXWS motif in the extra-cellular ligand-binding region [8,9]. The single copy EpoR gene is located on chromosome 19 and contains eight exons, where exons 1–5 code for the extracellular region, exon 6 the transmembrane, and exons 7–8 the cytoplasmic domain [10,11]. The latter two exons are thought to be the positive and negative regulatory regions of the receptor, respectively [12].

The interaction between Epo and its receptor initiates signal transduction pathways and ultimately Epo-induced cell proliferation leading to an increase in red cell mass. Epo induces homodimerization of the receptor molecule [5]. This causes the associated protein kinase Janus kinase 2 (JAK2) to autophosphorylate [13,14] (see Figure 1). Once activated, JAK2 phosphorylates at least some of the eight tyrosines present in the cytoplasmic domain of the EpoR [15]. Following phosphorylation of the EpoR, a number of other signal transduction proteins also become phosphorylated and initiation of the signal transduction pathways occurs. One such signal transducer and activator of transcription (STAT), the protein STAT5, associates with the tyrosines 343 and 401 of the receptor [16,17]. When STAT5 is phosphorylated it can be translocated to the nucleus where it initiates gene transcription [18]. In addition the binding of Epo to its receptor triggers other signaling cascades involving, for example, phosphatidylinositol 3-kinase (PI 3-kinase) [19] which binds to tyrosine 479 and the associated pathways involving PLCγ [20], Vav [21], c-fps/fes [22], RasGap [23], and MAPK [15], whose roles in signaling proliferation and differentiation remain to be defined. However, there is considerable functional redundancy in the tyrosines in a biological context but selective tyrosines may be uniquely important for erythroid development [24,25].

After addition of Epo, tyrosines in the cytoplasmic domain of the receptor become phosphorylated but after approximately 30 min they are no longer phosphorylated implicating the action of tyrosine phosphatases. The tyrosine phosphatase SHP-2 (also known as Syp, SHPTP-2) binds to the tyrosine 401 and stimulates cell proliferation [26]. In contrast SHP-1 (also known as HCP, SHPTP-1) binds at tyrosine 429 and inhibits cell proliferation, by the dephosphorylation of JAK2 [27], and cell differentiation [28]. If the negative regulatory system where cell proliferation is inhibited through SHP-1 was missing or nonfunctional then down-regulation would not occur and the EpoR would provide a continuous signal for cell proliferation and differentiation.

Under strictly controlled experimental conditions using serum free media the truncated EpoR appears to be hyporesponsive to Epo [15]. Damen and Krystal [15] also suggest that insulin growth factor-1 (IGF-1) contributes to the hyperresponsiveness to Epo when serum is present. It has recently been shown that insulin receptor substrate-2 (IRS-2) can be phosphorylated either by Epo or IGF-1 and the phosphorylated product can activate P1 3-kinase [29] and further downstream events. These alternative pathways lead to cell proliferation and could account for differences between serum free experiments and the in vivo situation in patients.

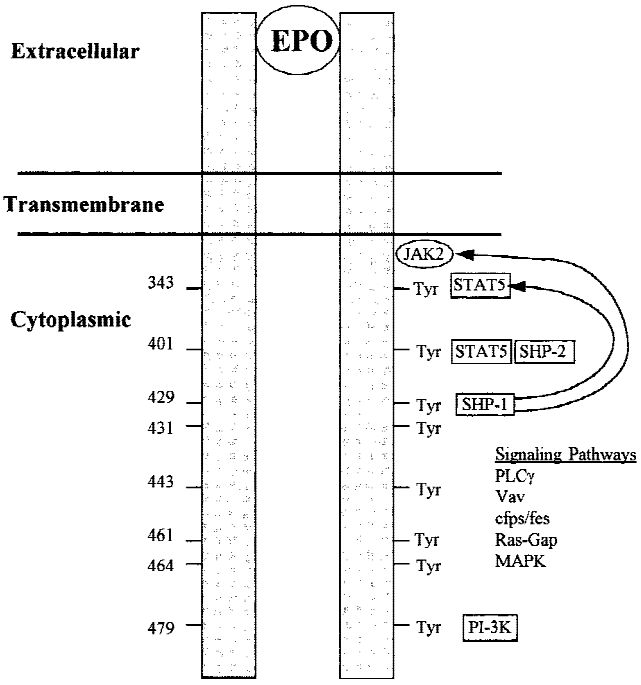

Fig. 1. Diagram of the erythropoietin receptor showing the tyrosines in the cytoplasmic region and sites of attachment of signal transduction proteins. Tyrosine residues are indicated by Tyr. Amino acid positions are noted on the left. The notation used is that of the mature 483 amino acid protein. Known mutated receptors lack between 59 and 84 terminal amino acids and therefore the negative regulatory 429 binding site is lost, but not at site 343 where proliferation is initiated. Some of the possible signaling proteins are indicated on the right.

The Erythropoietin Receptor in Hematological Disease

PV is characterized by clonal hematopoietic stem cell proliferation [30,31] and is associated with low serum Epo levels [32,33]. Culturing of erythroid progenitor cells from the blood of PV patients has indicated either erythroid colony formation in the absence of Epo [34,35] or increased sensitivity to Epo [36–38] This is maintained with serum-free conditions [39,40]. It has therefore been suggested that the erythropoietin receptor may play a role in the pathogenesis of PV. This idea is supported by the observation that in mice, a mutation in the extracellular domain of the EpoR results in the receptor

TABLE I. Investigations of the Erythropoietin Receptor in Disease*

Patient population	Tests on EpoR	Abnormality	Reference
Polycythemia vera (24)	RT-SSCP, sequencing (4)	None	43
Myeloproliferative disease, (PV 11, MF 5, ET 4)	Southern blot	None	44
Myeloproliferative disease (PV 4, ET 3, erythrocytosis 4)	Sequencing of exons 7 and 8	None	46
Myeloproliferative disease (6)	PCR, cloning and sequencing of DNA from BFU-E	None	45
Polycythemia vera (12)	Sequencing of EpoR	None	47
Myelodysplastic/myeloproliferative (3)	PCR, cloning and sequencing of DNA from BFU-E	C to T nt 955, exon 7 T to C nt 1024 exon 8, significance?	53
Myelodysplasia (8 RARS)	Southern blot	1 3kb 5′ deletion in one allelle (polymorphism?) (familial, present in sibling)	44
Erythroleukemia (10)	Southern blot and sequencing of EpoR	1 A to G nt 6148 polymorphism?	47
Diamond-Blackfan anemia (23)	Southern blot (7) SSCP (23)	None	54
Pure red cell aplasia (2)	Sequencing of exons 7 and 8	None	46

*PV, polycythemia; ET, essential thrombocythemia; MF, myelofibrosis; RARS, refractory anemia with ring sideroblasts. The number of patients examined in each study is shown in parentheses.

being constitutively activated [41,42] and deletions of the cytoplasmic domain have been found to increase Epo-mediated proliferation and cell survival [12,41].

However, no structural abnormalities of the EpoR have been detected [43,44] in PV and related myeloproliferative disorders. Consequently it has been suggested that point mutations of the EpoR may provide an explanation for acquired and congenital polycythemias. Several studies have failed to detect point mutations in the EpoR in patients with acquired polycythemia [43–47]. In retrospect it is not surprising that the EpoR has not been shown to be involved in the pathogenesis of PV. Erythroid progenitors from PV patients have been shown to be hypersensitive not only to Epo but also to a range of growth factors, and in fact, myeloid and megakaryocyte progenitors from these patients are also hypersensitive to growth factors [48,49]. It is likely that PV results from a somatic mutation of a multi-potent progenitor cell leading to dysregulated growth of a clone of abnormal cells; this is reviewed by Hinshelwood et al. [50]. Most recently it has been shown that deregulated expression of the inhibitor of apoptosis Bcl-x may contribute to the Epo-independent survival of erythroid cells in PV [51] and that the thrombopoietin receptor Mpl is expressed at a reduced level [52], again supporting the possibility of an abnormality leading to dysregulated growth.

Mutations of the erythropoietin receptor have been investigated as a possible cause of other related diseases. Two patients with thrombocytosis and ring sideroblasts were found to possess amino acid substitutions, but their relevance to the pathogenesis of the disease has not been defined [53]. In one case of myelodysplastic disorder with idiopathic sideroblastic anemia, a deletion at the 5′ region of the EpoR gene was detected, but was thought to be a polymorphism [44]. No abnormalities have been found in patients with Diamond-Blackfan syndrome or pure red cell aplasia [54,40]. In one of 10 cases of erythroleukemia, a single base change is reported in exon 8. However, this mutation was present in Epstein-Barr virus-derived cell lines from the patient and therefore did not appear to be related to the malignant clone, and transfection of the mutant receptor into BaF3 cells did not affect the response to Epo of the transfected cells. Therefore, it did not account for the disease phenotype [47]. Thus abnormalities of the erythropoietin receptor have not been shown to have a role in the pathogenesis of any of the above disorders (see Table I).

The Erythropoietin Receptor in Idiopathic Erythrocytosis

In idiopathic erythrocytosis, there is an increase in the red cell mass but the patient does not fulfill the other criteria for PV [55]. A family history may be elicited. Studies of affected families have revealed seven EpoR mutations, which cause the formation of a stop codon [56–62] (see Table II). Initially, these mutations were believed to be rare, but the same mutation has been shown to have arisen twice independently [63]. The G6002A mutation was first described in a Finnish family whose members suffered from familial erythrocytosis associated with low serum Epo levels [56]. The same mutation has been detected de novo in a teenage boy of English descent [63]. This case suggests that it may be

TABLE II. Summary of Reported Erythropoietin Receptor Mutations in Idiopathic Erythrocytosis

Mutation	Nucleotide position	Effect of mutation	Truncation	Reference
G to T	5959	Stop codon	84 aa	61
C to G	5964	Stop codon	82 aa	58
Insertion-T	5967	Frameshift	65 aa	60
Insertion-G	5974	Frameshift	64 aa	57
Deletion	5985–5991	Frameshift	59 aa	62,60
C to T	5986	Stop codon	74 aa	59
G to A	6002	Stop codon	70 aa	56,63
A to G	6146	Missense	Point Mutation	47
C to T	6148	Missense	Point Mutation	65

worth looking further for EpoR mutations in cases of idiopathic erythrocytosis when the plasma Epo is low.

Hypersensitivity to Epo has been exhibited by cells transfected with mutant 5974inG and G6002A EpoR molecules [57], thereby suggesting that the ability of the erythroid progenitor cells to grow in the absence of exogenous Epo is a consequence of these mutations. However, in one case [58], an unaffected 2-year-old daughter, also had the mutation nt5964, C to G. This girl's erythroid progenitor cells only demonstrated slightly increased sensitivity in vitro unlike the markedly increased sensitivity of her affected relatives. More recent follow up on this child [46] suggests that she has a slight but significant increase in Epo sensitivity but still a normal hemoglobin, which suggests that in some cases other genetic factors may modulate the response to the EpoR mutation. In addition, two point mutations A to G at nt 6146 and C to T at nt 6148 have been detected and in both cases, transfection of the mutant receptor into BaF3 cells did not affect the response of the transfected cells to Epo [65,47]. These point mutations do not seem to account for the disease state.

The seven described EpoR mutations are clustered in a 44 base pair (bp) region and result in the formation of a stop codon. Consequently, between 59 and 82 amino acids are lost from the terminus of the receptor molecule (see Table II) [56–63]. This region includes tyrosine 429, the binding site of the negative regulator SHP-1 [27]. Gene transcription can be initiated via the JAK2/STAT5 pathway at tyrosine 343. Loss of the SHP-1 binding site, and hence function, would prevent dephosphorylation of JAK2 (Fig. 1). Prolonged STAT5 activation has been observed in cells transfected with a truncated mutant EpoR, suggesting that enhanced STAT5 DNA binding activity may play a role in the pathogenesis of erythrocytosis [66].

Other Explanations for Idiopathic Erythrocytosis

Idiopathic erythrocytosis is a heterogenous disease and EpoR mutations do not explain all cases. There has been a report of congenital polycythemia in Chuvashia that is associated with high Epo levels, and no linkage of the disorder with the EpoR gene could be established [67]. Therefore, cases exist in which the cause of erythrocytosis remains unknown. If Epo levels are low and the EpoR is intact, then mutations of other genes involved in the signaling pathway of the EpoR must be considered. It is conceivable that JAK2, STAT5, or other transcription factors may be abnormal in patients with idiopathic erythrocytosis.

Epo gene expression is under the control of *cis*-acting sequences 5′ and 3′ to the gene. A hypoxia-responsive element at the 3′ end of the Epo gene includes a minimal enhancer region of between 24 and 50 bp. This area is bound by the transcription factors, hypoxia-inducible factor-1 (HIF-1) and hepatic nuclear factor-4 (HNF-4). Interaction between these factors and the transcriptional activator p300 provides a mechanism for the induction of Epo gene expression [68]. It is theoretically possible that a mutation of the 3′ hypoxia-responsive element could constitutively activate the Epo gene causing erythrocytosis due to the presence of an elevated serum Epo level. We have investigated patients with idiopathic erythrocytosis and related myeloproliferative disorders for such mutations. Polymorphisms have been found in the 3′enhancer region of the Epo gene but none of these have been found to account for the disease phenotype [69]. The 5′ promoter region [70] of the Epo gene may also harbor mutations and should be considered in the future in patients with idiopathic erythrocytosis and high plasma Epo levels. Transcription factors involved in the response to hypoxia should also be considered. For instance, abnormalities of HIF-1 binding to the Epo gene could lead to an altered response by the oxygen sensing pathway which could account for some of the disease presentations [71].

CONCLUSIONS

In cases of unexplained erythrocytosis associated with selective expansion of the red cell lineage and low serum Epo, mutations in the EpoR may provide a mechanism for the overproduction of erythroid cells and hypersensitivity to Epo and explain the low resulting serum Epo levels. Investigation of all such cases should include a search for mutations of at least exons 7 and 8 of the EpoR. The pathogenesis of the acquired myeloproliferative disorders remains to be elucidated.

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